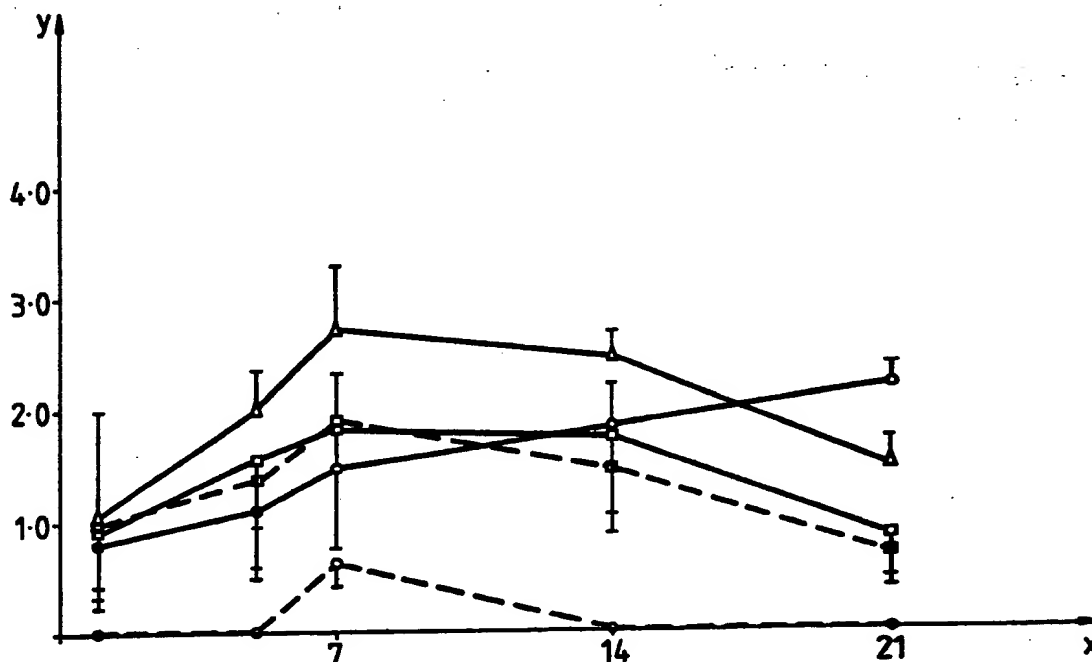




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(54) Title: EXPRESSION OF RECOMBINANT PROTEINS IN ATTENUATED BACTERIA



## (57) Abstract

An attenuated bacterium which is capable of expressing a heterologous protein, the expression of the heterologous protein being under the control of a promoter whose activity is induced by anaerobic conditions, can be used as a vaccine. A suitable promoter is the *nirB* promoter.

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EXPRESSION OF RECOMBINANT PROTEINS IN ATTENUATED BACTERIA

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This invention relates to attenuated bacteria capable of expressing a heterologous protein, to their preparation and to vaccines containing them.

5 Virulent strains of Salmonella can be attenuated by introducing specific mutations into genes required for survival and growth in vivo. Attenuated variants which establish self limiting, clinically insignificant infections can be considered as potential live oral vaccines against  
10 Salmonella infections. Ty21a is an attenuated variant of Salmonella typhi, which harbours mutations in galE and other unknown attenuating lesions, and is licensed for use in many countries as a live oral typhoid vaccine.

More recently genetically defined Salmonella  
15 strains harbouring individual specific mutants in different genes have been tested as experimental oral vaccines in several target species. For example, Salmonella aro mutants, which have an auxotrophic requirement for several aromatic compounds, have been shown to be effective oral  
20 vaccines in mice, sheep, cattle, chickens and more recently they have been shown to be attenuated and immunogenic in volunteers. Salmonella double aro mutants are disclosed in EP-A-0322237. Salmonella cya crp double mutants are also effective oral vaccines.

25 As well as being vaccines in their own right against salmonellosis, attenuated Salmonellae can be considered as carriers of heterologous antigens to the immune oral system. This is because Salmonellae can be delivered via the oral route and are potent immunogens being  
30 able to stimulate systemic and local cellular and antibody responses. Heterologous antigens from bacteria, viruses and parasites can be delivered to the host using Salmonella vaccines.

One potentially serious drawback in using these  
35 live vaccines for antigen delivery relates to problems with the stability of the foreign antigen expression in vivo.

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Unregulated expression of high levels of a foreign protein in bacteria from multiple copy plasmids usually results in rapid loss of the plasmid or expressed gene from the cells. This problem can be controlled in fermenters by using  
5 inducible promoter systems such as trp or lac to allow the controlled induction of gene expression when the appropriate biomass has been achieved. Obviously these promoters can not be induced by exogenously applied inducers such as PP or IPTG when bacteria are growing in host tissues during the  
10 self-limited growth following vaccination.

In vivo plasmid instability during vaccination with live bacterial vectors has in fact been reported by many workers (Maskell et al, Microb.Path 2, 295-305, 1987; Nakayama et al, Bio/technology 6, 693-697, 1988; Tite et al,  
15 Immunology 70, 540-546, 1990). A number of approaches have been taken to overcome the problem including the use of integration systems for expression of the heterologous antigen from the bacterial chromosome (Hone et al, Microbiol. Path. 5, 407-418, 1988; Strugnelli et al, Gene 88,  
20 57-63, 1990). However, this approach is only suitable for use with some antigens since expression levels are often quite low (Maskell et al, 1987). Nakayama et al described the use of linking an essential gene to the expression plasmid for stabilizing in vivo expression. Although this  
25 is a highly effective approach, it does not prevent the generation of plasmid free variants but simply ensures they do not survive. Further stable but constitutive high level expression of a foreign antigen in a Salmonella vaccine strain could slow down the growth rate and hence potentially  
30 effect the immunogenicity of the live vaccine.

According to the present invention, there is provided an attenuated bacterium which is capable of expressing a heterologous protein, the expression of the heterologous protein being under the control of a promoter  
35 whose activity is induced by anaerobic conditions.

Stable expression of the heterologous protein can

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be obtained in vivo. The attenuated bacterium can therefore be used as a vaccine. Any suitable bacterium may be employed, for example a Gram-negative bacterium. Some Gram-negative bacteria such as Salmonella invade and grow within eucaryotic cells and colonise mucosal surfaces.

The attenuated bacterium may therefore be selected from the genera Salmonella, Bordetella, Vibrio, Haemophilus, Neisseria and Yersinia. Alternatively, the attenuated bacterium may be an attenuated strain of Enterotoxigenic Escherichia coli. In particular the following species can be mentioned: S. typhi - the cause of human typhoid; S. typhimurium - the cause of salmonellosis in several animal species; S. enteritidis - a cause of food poisoning in humans; S. choleraesuis - a cause of salmonellosis in pigs; Bordetella pertussis - the cause of whooping cough; Haemophilus influenzae - a cause of meningitis; Neisseria gonorrhoeae - the cause of gonorrhoea; and Yersinia - a cause of food poisoning.

Attenuation of the bacterium may be attributable to a non-reverting mutation in a gene in the aromatic amino acid biosynthetic pathway of the bacterium. There are at least ten genes involved in the synthesis of chorismate, the branch point compound in the aromatic amino acid biosynthetic pathway. Several of these map at widely differing locations on the bacterial genome, for example aroA (5-enolpyruvylshikimate-3-phosphate synthase), aroC (chorismate synthase), aroD (3-dihydroquinate dehydratase) and aroE (shikimate dehydrogenase). A mutation may therefore occur in the aroA, aroC, aroD or aroE gene.

Preferably, however, an attenuated bacterium harbours a non-reverting mutation in each of two discrete genes in its aromatic amino acid biosynthetic pathway. Such bacteria are disclosed in EP-A-0322237. Double aro mutants which are suitable are aroA aroC, aroA aroD and aroA aroE mutant bacteria. Other bacteria having mutations in other combinations of the aroA, aroC, aroD and aroE genes are

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however useful. Particularly preferred are Salmonella double aro mutants, for example double aro mutants of S.typhi or S.typhimurium, in particular aroA aroC, aroA aroD and aroA aroE mutants.

5 Alternatively, the attenuated bacterium may harbour a non-reverting mutation in a gene concerned with the regulation of one or more other genes (EP-A-0400958). Preferably the mutation occurs in the ompR gene or another gene involved in regulation. There are a large number of 10 other genes which are concerned with regulation and are known to respond to environmental stimuli (Ronson et al, Cell 49, 579-581).

This type of attenuated bacterium may harbour a second mutation in a second gene. Preferably the second gene is a gene encoding for an enzyme involved in an essential biosynthetic pathway, in particular genes involved in the pre-chorismate pathway, in particular genes involved 15 of aromatic compounds. The second mutation is therefore preferably in the aroA, aroC or aroD gene.

Another type of attenuated bacterium is one in which attenuation in DNA of the bacterium is one in 20 which attenuation is brought about by the presence of a non-reverting mutation in response to environmental stress, or which regulates the expression of DNA encoding, a protein that is produced in response to environmental stress. Such 25 bacteria are disclosed in WO 91/15572. The non-reverting mutation may be a deletion, insertion, inversion or substitution. A deletion mutation may be generated using a transposon.

Examples of proteins that are produced in response 30 to environmental stress include heat shock proteins (which are produced in response to a temperature increase above 42°C); nutrient deprivation proteins (which are produced in response to levels of essential nutrients such as phosphates or nitrogen which are low that which the microorganism 35 requires to survive); toxic stress proteins (which are produced in response to toxic compounds such as dyes, acids

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or possibly plant exudates); or metabolic disruption proteins (which are produced in response to fluctuations in for example ion levels affecting the microorganisms ability to osmoregulate, or vitamin or co-factor levels such as to 5 disrupt metabolism).

Preferably a heat shock protein is the one encoded by the htrA gene, also characterised as degP. Other proteins are encoded by genes known to be involved in the stress response such as grpE, groEL, (moPA), dnaK, groES, 10 lon and dnaJ. There are many other proteins encoded by genes which are known to be induced in response to environmental stress (Ronson *et al.*, Cell 49, 579-581). Amongst these the following can be mentioned: the ntrB/ntrC system of E. coli, which is induced in response to nitrogen 15 deprivation and positively regulates glnA and nifH (Buck *et al.*, Nature 320, 374-378, 1986; Hirschman *et al.*, Proc. Natl. Acad. Sci. USA, 82, 7525, 1985; Nixon *et al.*, Proc. Natl. Acad. Sci. USA 83, 7850-7854, 1986, Reitzer and Magasanik, Cell, 45, 785, 1986); the phoR/phoB system of E. 20 coli which is induced in response to phosphate deprivation (Makino *et al.*, J. Mol. Biol. 192, 549-556, 1986b); the cpxA/sfrA system of E. coli which is induced in response to dyes and other toxic compounds (Albin *et al.*, J. Biol. Chem. 261 4698, 1986; Drury *et al.*, J. Biol. Chem. 260, 4236-4272, 25 1985). An analogous system in Rhizobium is dctB/dctD, which is responsive to 4C-dicarboxylic acids (Ronson *et al.*, J. Bacteriol. 169, 2424 and Cell 49, 579-581, 1987). A virulence system of this type has been described in Agrobacterium. This is the virA/virG system, which is 30 induced in response to plant exudates (le Roux *et al.*, EMBO J. 6, 849-856, 1987; Stachel and Zambryski., Am. J. Vet. Res 45, 59-66, 1986; Winans *et al.*, Proc. Natl. Acad. Sci. USA, 83, 8278, 1986). Similarly the bvgC-bvgA system in Bordetella pertussis (previously known as vir) regulates the 35 production of virulence determinants in response to fluctuations in Mg<sup>2+</sup> and nicotinic acid levels (Arico *et al.*,

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1989, Proc. Natl. Acad. Sci. USA 86, 6671-6675).

For use in the form of a live vaccine, an attenuated bacterium should not revert back to the virulent state. The probability of this happening with a mutation in a single DNA sequence is considered to be small. However, the risk of reversion occurring with a bacterium attenuated by the presence of mutations in each of two discrete DNA sequences is considered to be insignificant. A preferred attenuated bacterium is therefore one in which attenuation is brought about by the presence of a mutation in a DNA sequence which encodes, or which regulates the expression of DNA encoding, a protein that is produced in response to environmental stress and by the presence of a mutation in a second DNA sequence.

The second DNA sequence preferably encodes an enzyme involved in an essential auxotrophic pathway or is a sequence whose product controls the regulation of osmotically responsive genes, i.e. ompR, (Infect and Immunity 1989 2136-2140). Most preferably, the mutation is in a DNA sequence involved in the aromatic amino acid biosynthetic pathway, more particularly the DNA sequences encoding aroA, aroC or aroD.

Attenuated bacteria may be constructed by the introduction of a mutation into the DNA sequence by methods known to those skilled in the art (Maniatis, Molecular Cloning and Laboratory Manual, 1982). Non-reverting mutations can be generated by introducing a hybrid transposon TnphoA into, for example, S.typhimurium strains. TnphoA can generate enzymatically active protein fusions of alkaline phosphatase to periplasmic or membrane proteins. The TnphoA transposon carries a gene encoding kanamycin resistance. Transductants are selected that are kanamycin resistant by growing colonies on an appropriate selection medium.

Alternative methods include cloning the DNA sequence into a vector, e.g. a plasmid or cosmid, inserting



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a selectable marker gene into the cloned DNA sequence, resulting in its inactivation. A plasmid carrying the inactivated DNA sequence and a different selectable marker can be introduced into the organism by known techniques (Maniatis, Molecular Cloning and Laboratory Manual, 1982). It is then possible by suitable selection to identify a mutant wherein the inactivated DNA sequence has recombined into the chromosome of the microorganism and the wild-type DNA sequence has been rendered non-functional in a process known as allelic exchange. In particular, the vector used is preferably unstable in the microorganism and will be spontaneously lost. The mutated DNA sequence on the plasmid and the wild-type DNA sequence may be exchanged by a genetic cross-over event. Additional methods eliminate the introduction of foreign DNA into vaccine strains at the site of mutations and the introduction of antibiotic resistant markers into the strains.

The heterologous antigen which an attenuated bacterium is capable of expressing may for example comprise an antigenic determinant of a pathogenic organism. The antigen may be derived from a virus, bacterium, fungus, yeast or parasite. The heterologous protein therefore typically comprises an antigenic sequence derived from a virus, bacterium, fungus, yeast or parasite. More especially, the antigenic sequence may be derived from a type of human immunodeficiency virus (HIV) such as HIV-1 or HIV-2, hepatitis A or B virus, human rhinovirus such as type 2 or type 14, herpes simplex virus, poliovirus type 2 or 3, foot-and-mouth disease virus, influenza virus, coxsackie virus, the cell surface antigen CD4 and Chlamydia trachomatis. The antigen may comprise the CD4 receptor binding site from HIV, for example from HIV-1 or -2. Other useful antigens include E. coli heat labile toxin B subunit (LT-B), E. coli K88 antigens, P.69 protein from B. pertussis, tetanus toxin fragment C and antigens of flukes, mycoplasma, roundworms, tapeworms, rabies virus and

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rotavirus.

A preferred promoter for use in controlling the expression of the heterologous protein is the nirB promoter. The nirB promoter has been isolated from E. coli, where it directs expression of an operon which includes the nitrite reductase gene nirB (Jayaraman *et al*, J. Mol. Biol. 196, 781-788, 1987), and nirD, nirC and cysG (Peakman *et al*, Eur. J. Biochem. 191, 315-323, 1990). It is regulated both by nitrite and by changes in the oxygen tension of the environment, becoming active when deprived of oxygen (Cole, Biochim. Biophys. Acta, 162, 356-368, 1968). Response to anaerobiosis is mediated through the protein FNR, acting as a transcriptional activator, in a mechanism common to many anaerobic respiratory genes.

By deletion and mutational analysis the part of the promoter which responds solely to anaerobiosis has been isolated and by comparison with other anaerobically-regulated promoters a consensus FNR-binding site was identified (Bell *et al*, Nucl. Acids. Res. 17, 3865-3874, 1989; Jayaraman *et al*, Nucl. Acids Res. 17, 135-145, 1989). It was also shown that the distance between the putative FNR-binding site and the -10 homology region is critical (Bell *et al*, Molec. Microbiol. 4, 1753-1763, 1990). It is therefore preferred to use only that part of the nirB promoter which responds solely to anaerobiosis. As used herein references to the nirB promoter refer to the promoter itself or a part or derivative thereof which is capable of promoting expression of a coding sequence under anaerobic conditions. The sequence which we have in fact used and which contains the nirB promoter is:

AATTCAGGTAAATTTGATGTACATCAAATGGTACCCCTTGCTGAATCGTTAAGGTA  
GGCGGTAGGGCC

An attenuated bacterium according to the present invention may be prepared by transforming an attenuated bacterium with a DNA construct comprising a promoter whose activity is induced by anaerobic conditions, such as the

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nirB promoter, operably linked to a DNA sequence encoding a heterologous protein. Any suitable transformation technique may be employed, such as electroporation. In this way, an attenuated bacterium capable of expressing a protein  
5 heterologous to the bacterium may be obtained. A culture of the attenuated bacterium may be grown under aerobic conditions. A sufficient amount of the bacterium is thus prepared for formulation as a vaccine, with minimal expression of the heterologous protein occurring.

10 The DNA construct is typically a replicable expression vector comprising the nirB promoter operably linked to a DNA sequence encoding the heterologous protein. The nirB promoter may be inserted in an expression vector, which already incorporates a gene encoding the heterologous  
15 protein, in place of the existing promoter controlling expression of the protein. The expression vector should of course be compatible with the attenuated bacterium into which the vector is to be inserted.

The expression vector is provided with appropriate  
20 transcriptional and translational control elements including, besides the nirB promoter, a transcriptional termination site and translational start and stop codons. An appropriate ribosome binding site is provided. The vector typically comprises an origin of replication and, if  
25 desired, a selectable marker gene such as an antibiotic resistance gene. The vector may be a plasmid.

An attenuated bacterium of the invention can be used as a vaccine. The vaccine comprises a pharmaceutically acceptable carrier or diluent and, as active ingredient, the  
30 attenuated bacterium.

The vaccine is advantageously presented in a lyophilised form, for example in a capsular form, for oral administration to a patient. Such capsules may be provided with an enteric coating comprising, for example, Eudragat  
35 "S", Eudragate "L", Cellulose acetate, cellulose phthalate or hydroxypropylmethyl cellulose. These capsules may be

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used as such, or alternatively, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is advantageously effected in a buffer at a suitable pH to ensure the viability of the organisms. In order to protect the attenuated bacteria and the vaccine from gastric acidity, a sodium bicarbonate preparation is advantageously administered before each administration of the vaccine. Alternatively, the vaccine may be prepared for parenteral administration, intranasal administration or intramammary.

The attenuated bacterium of the invention may be used in the prophylactic treatment of a host, particularly a human host but also possibly an animal host. An infection caused by a microorganism, especially a pathogen, may therefore be prevented by administering an effective dose of an attenuated bacterium according to the invention. The bacterium then expresses a heterologous protein capable of raising antibody to the microorganism. The dosage employed will be dependent on various factors including the size and weight of the host, the type of vaccine formulated and the nature of the heterologous protein. However, for attenuated S.typhi a dosage comprising the oral administration of from  $10^9$  to  $10^{11}$  S.typhi organisms per dose is generally convenient for a 70kg adult human host.

The following Example illustrates the invention. In the accompanying drawings:

Figures 1 to 4 show the abilities of isolates of S.typhimurium to grow in vivo in the liver, spleen, Peyer's patches and mesenteric lymph nodes respectively of BALB/c mice. The x-axis denotes days after infection, the y-axis denotes  $\log_{10}$  viable organisms per organ,  $\Delta$  denotes isolate BRD509,  $\square$  denotes isolate BRD847,  $\circ$  denotes isolate BRD743, — denotes no ampicillin and ---- denotes ampicillin added.

Figure 5 shows anti-tetanus toxin fragment C titres of mouse sera. The x-axis shows the types of bacteria used

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of mouse sera. The x-axis shows the types of bacteria used to challenge the mice. The number of doses is shown in brackets. The y-axis denotes absorbance readings at 492nm.

# EXAMPLE

## 5 Construction of pTETnir15

Expression plasmid pTETnir15 was constructed from pTETtac115 (Makoff *et al*, Nucl. Acids Res. 17 10191-10202, 1989) by replacing the EcoRI-ApaI region (1354bp) containing the lacI gene and tac promoter with the following pair of

10 oligos 1 and 2:

Oligo-1 5'-AATTCAGGTAAATTTGATGTACATCAAATGGTACCCCTTGCTGAATC

Oligo-2 3'-GTCCATTTAAACTACATGTAGTTTACCATGGGGAACGACTTAG

GTTAAGGTAGGCGGTAGGGCC-3'

CAATTCCATCCGCCATC-5'

15 The oligonucleotides were synthesized on a Pharmacia Gene Assembler and the resulting plasmids confirmed by sequencing (Makoff *et al*, Bio/Technology 7, 1043-1046, 1989).

## Construction of SL1334 *aroA aroD* harbouring pTETnir15

20 In order to construct a Salmonella vaccine strain expressing tetanus toxin fragment C under the control of the nirB promoter, an intermediate strain, S.typhimurium LB5010 ( $r^{-}m^{+}$ ) (Bullas and Ryo, J. Bact. 156, 471-474, 1983), was transformed with pTETnir15. Colonies expressing fragment C  
25 were detected by antibiotic selection followed by colony immunoblotting with anti-tetanus toxin fragment C sera. Colonies were grown overnight on nitrocellulose filters aerobically and then induced by incubating under anaerobic conditions for four hours prior to immunoblotting. One  
30 strain that was stably expressing fragment C was used to prepare plasmid DNA. This was used to transform an isolate of S.typhimurium SL1344 *aroA aroD* designated BRD509 by electroporation. A strain that was stably expressing  
fragment C (checked by immunoblotting as described above)  
35 was chosen for the in vivo studies and was designated

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Comparison of in vivo kinetics of BRD743 and BRD847 in BALB/c mice

The ability of BRD743 (BRD509 harbouring pTET85) and of BRD847 to grow in vivo was compared after oral  
5 administration to BALB/c mice. pTET85 was constructed from pTETtac115 (Makoff et al, Nucl. Acids Res. 17, 10191-10202, 1989) by deleting the 1.2kb EcoRI fragment carrying the lacI gene. This resulted in the constitutive expression of fragment C in Salmonella strains. Numbers of bacteria were  
10 enumerated in livers, spleens, Peyers patches and mesenteric lymph nodes. The bacteria isolated from mice were also assessed for their ability to grow on plates containing ampicillin as an indicator of the percentage of organisms still retaining the plasmid expressing fragment C. The  
15 results are shown in Figures 1 to 4.

When similar initial numbers of organisms ( $5 \times 10^9$ ) were used to infect mice it was found that both BRD743 and 847 were able to invade into and persist in all the murine tissues examined but at a lower level than BRD509. However,  
20 the interesting feature is that the number of ampicillin resistant organisms obtained from mice infected with BRD743 decreases rapidly and all organisms recovered were ampicillin sensitive by day 14. This indicates that in vivo selection rapidly results in the loss of pTET85 from the  
25 Salmonella vaccine strain. In contrast, counts with and without ampicillin for BRD847 were essentially the same for the time the infections were monitored. This demonstrates the added advantage of pTETnir15 in the S.typhimurium vaccine strain resulting in organisms with the potential to  
30 express fragment C in vivo for a longer period of time with obvious advantages in terms of immunogenicity.

Immunisation of BALB/c mice using Salmonella strains harbouring pTET85 (BRD743) r pTETnir15 (BRD847)

Groups of twenty mic were incubat d orally with

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5 x 10<sup>9</sup> cells per mouse of either BRD743, BRD847 or BRD509. On day 25 serum was collected from all mice and analysed by ELISA for anti-tetanus antibodies. All mice vaccinated with BRD847 had detectable anti-fragment C antibody at 25 days whereas those vaccinated with BRD743 or BRD509 did not (Fig. 5). On day 25 ten mice from each group were boosted by oral inoculation with a similar amount of homologous organisms. ELISA analysis of the serum taken from these mice at day 46 showed that the anti-fragment C responses had been boosted for groups inoculated with BRD743 and BRD847. The titres for those mice boosted with BRD847 was significantly higher than for those mice boosted with BRD743. Mice boosted orally with BRD509 failed to produce a detectable antibody response to fragment C.

15 Tetanus toxin challenge of mice orally immunised with BRD847 and 743

The mice vaccinated orally with BRD743, 847 and 509 were tested for immunity against tetanus toxin challenge after one or two doses of the immunising strain. Groups of 20 twenty mice received one single oral dose of 5 x 10<sup>9</sup> organisms and groups of ten mice were challenged on day 25 with 500 50% lethal doses of tetanus toxin (see Table 1). Mice vaccinated with BRD847 were completely protected against challenge after a single oral dose whereas those 25 vaccinated with BRD743 were only partially protected (2/10 survivors). The remaining groups of 10 mice received a second dose of organisms (5 x 10<sup>9</sup>) on day 25 and were challenged on day 46 (after the 1st dose). Again mice immunised with BRD847 were completely protected after 30 challenge with tetanus toxin whereas those immunised with BRD743 were only partially protected (5/10). Mice immunised with 1 or 2 doses of BRD509 and challenged with tetanus toxin all died. BRD847 is an effective single dose oral vaccine against tetanus toxin challenge in mice. Groups of 35 mice were also challenged with tetanus toxin after receiving

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1 and 2 intravenous doses of  $10^5$  organisms of BRD847 and BRD743. All mice were fully protected against challenge with tetanus toxin after 1 or 2 doses of vaccine strain.

TABLE 1

5 Oral immunisation of mice against tetanus using  
S.typhimurium SL1344 aroA aroD pTET85 and S.typhimurium  
SL1344 aroA aroD pTETnir15

<u>Vaccine</u>	<u>Dose</u>	<u>No. Doses</u>	<u>No. of mice</u> <u>surviving tetanus</u> <u>challenge</u>
10			
SL1344 <u>aroA aroD</u>	$8.6 \times 10^9$	1	0/10
(BRD509)	$7.4 \times 10^9$	2	0/10
SL1344 <u>aroA aroD</u>	$6.4 \times 10^9$	1	2/10
pTET85 (BRD743)	$8.2 \times 10^9$	2	5/10
15 SL1344 <u>aroA aroD</u>	$9.5 \times 10^9$	1	10/10
pTETnir15 (BRD847)	$7.5 \times 10^9$	2	9/9



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CLAIMS

1. An attenuated bacterium which is capable of expressing a heterologous protein, the expression of the heterologous protein being under the control of a promoter whose activity is induced by anaerobic conditions.

2. An attenuated bacterium according to claim 1, which is an attenuated strain of Salmonella.

3. An attenuated bacterium according to claim 2, which is an attenuated strain of Salmonella typhi or Salmonella typhimurium.

4. An attenuated bacterium according to any one of the preceding claims, in which attenuation is attributable to a non-reverting mutation in a gene in the aromatic amino acid biosynthetic pathway.

5. An attenuated bacterium according to any one of the preceding claims, in which attenuation is attributable to a non-reverting mutation in DNA of the bacterium which encodes, or which regulates the expression of DNA encoding, a protein that is produced in response to environmental stress.

6. An attenuated bacterium according to claim 4, which harbours a non-reverting mutation in each of two discrete genes in its aromatic amino acid biosynthetic pathway.

7. An attenuated bacterium according to claim 6, which is an aroA aroC, aroA aroD or aroA aroE mutant.

8. An attenuated bacterium according to any one of the preceding claims, in which the promoter is the nirB promoter.

9. An attenuated bacterium according to any one of the preceding claims, in which the heterologous protein comprises an antigenic sequence derived from a virus, bacterium, fungus, yeast or parasite.

10. An attenuated bacterium according to claim 9, in which the heterologous protein is the P.69 protein from Bordetella pertussis or is the toxin fragment C.

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11. A process for the preparation of an attenuated bacterium as claimed in any one of the preceding claims, which process comprises transforming an attenuated bacterium with a DNA construct comprising a said promoter operably  
5 linked to a DNA sequence encoding a said heterologous protein.

12. A vaccine comprising a pharmaceutically acceptable carrier or diluent and, as active ingredient, an attenuated bacterium as claimed in any one of claims 1 to  
10 10.

Fig. 1.

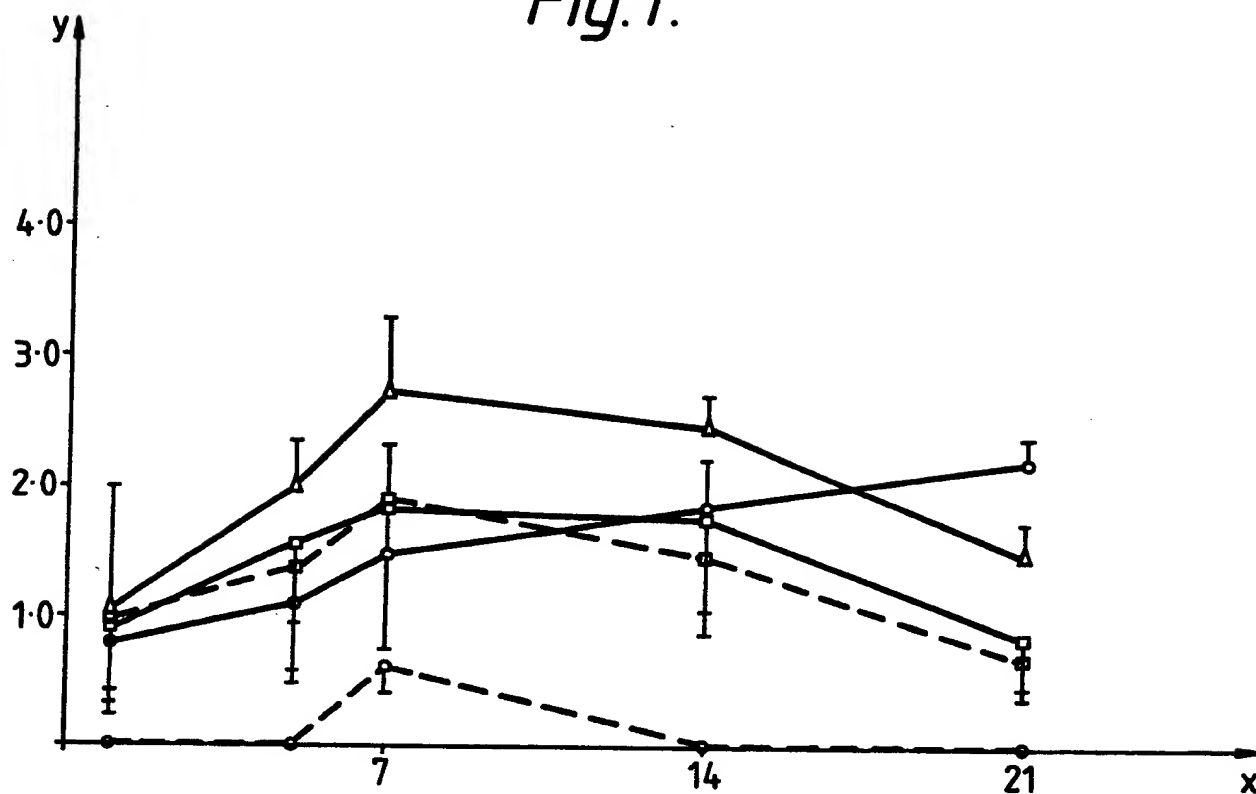


Fig. 2.

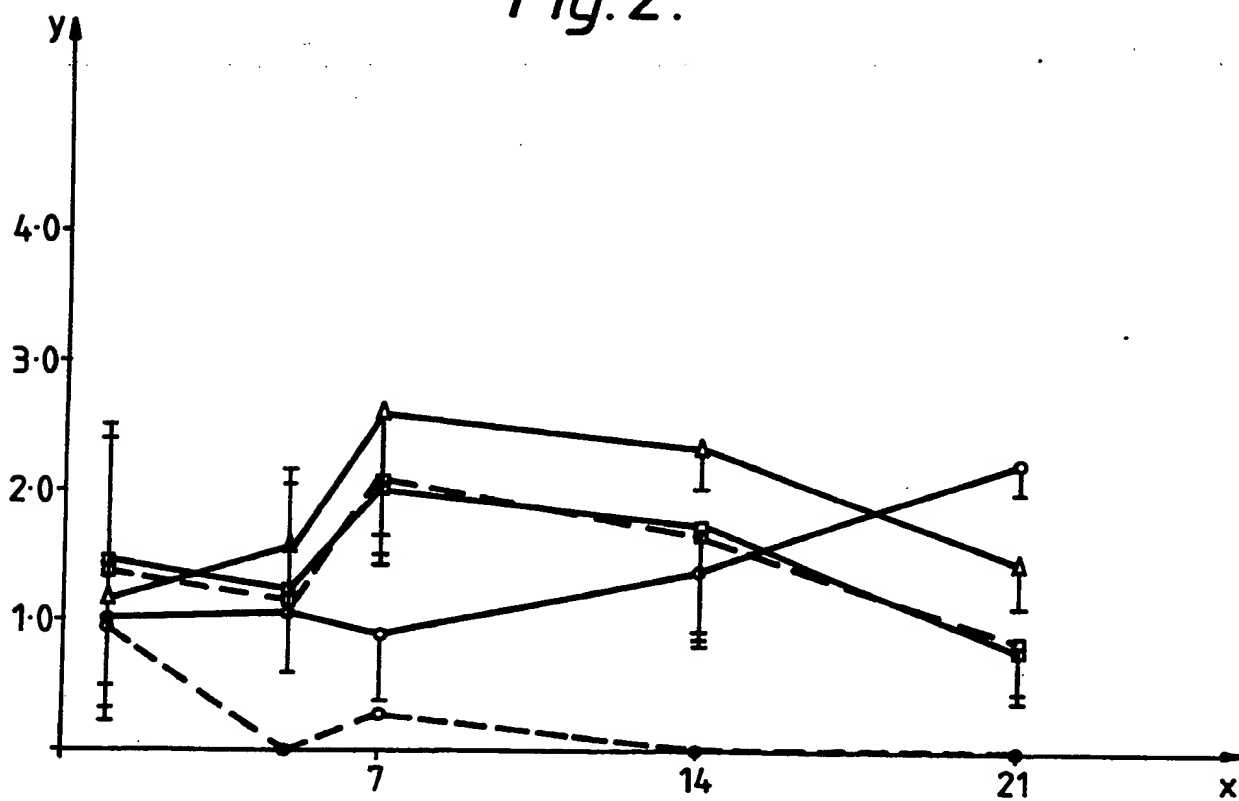


Fig.3.

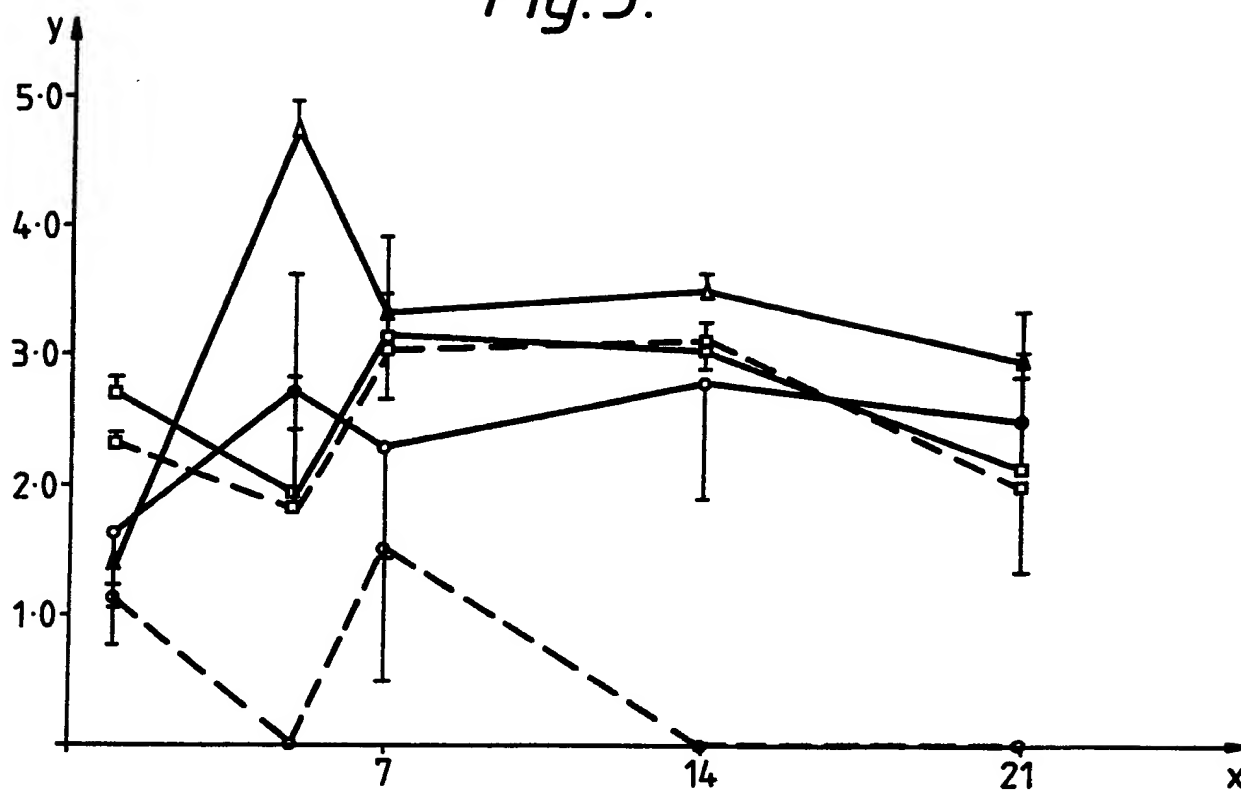
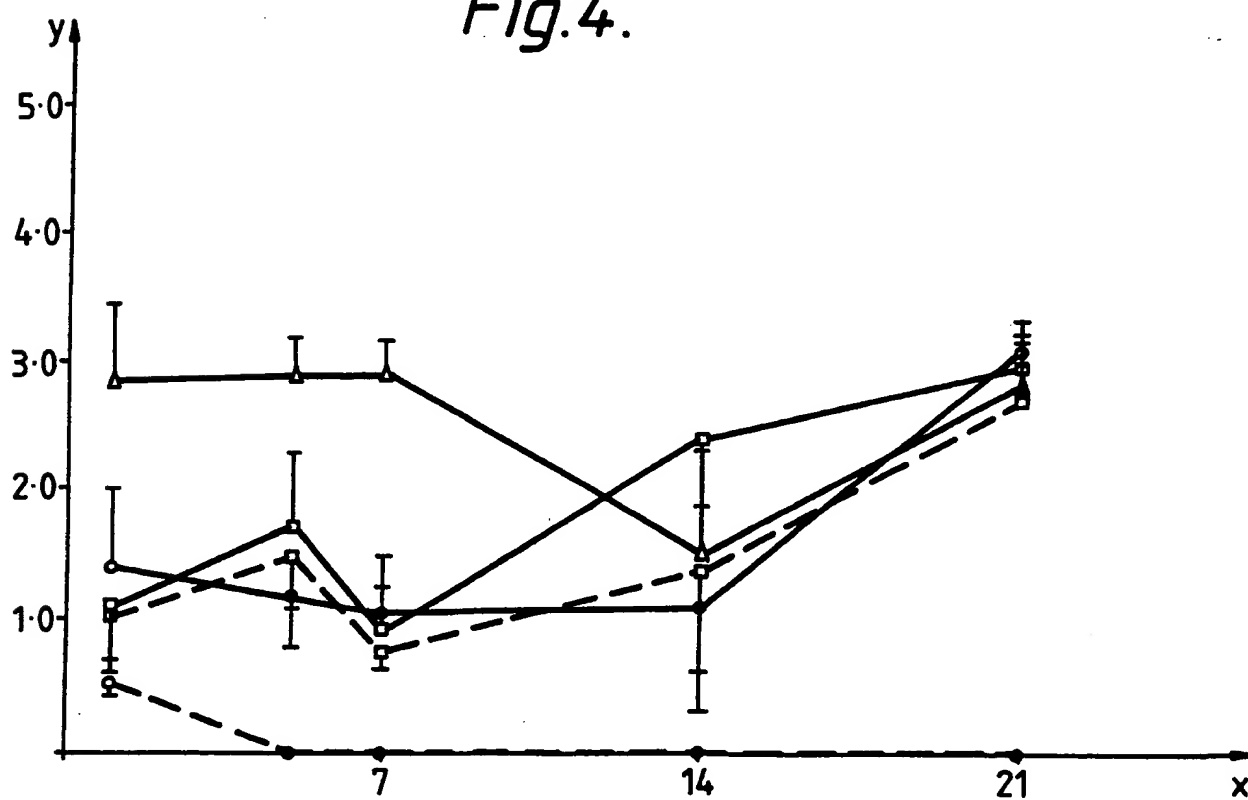
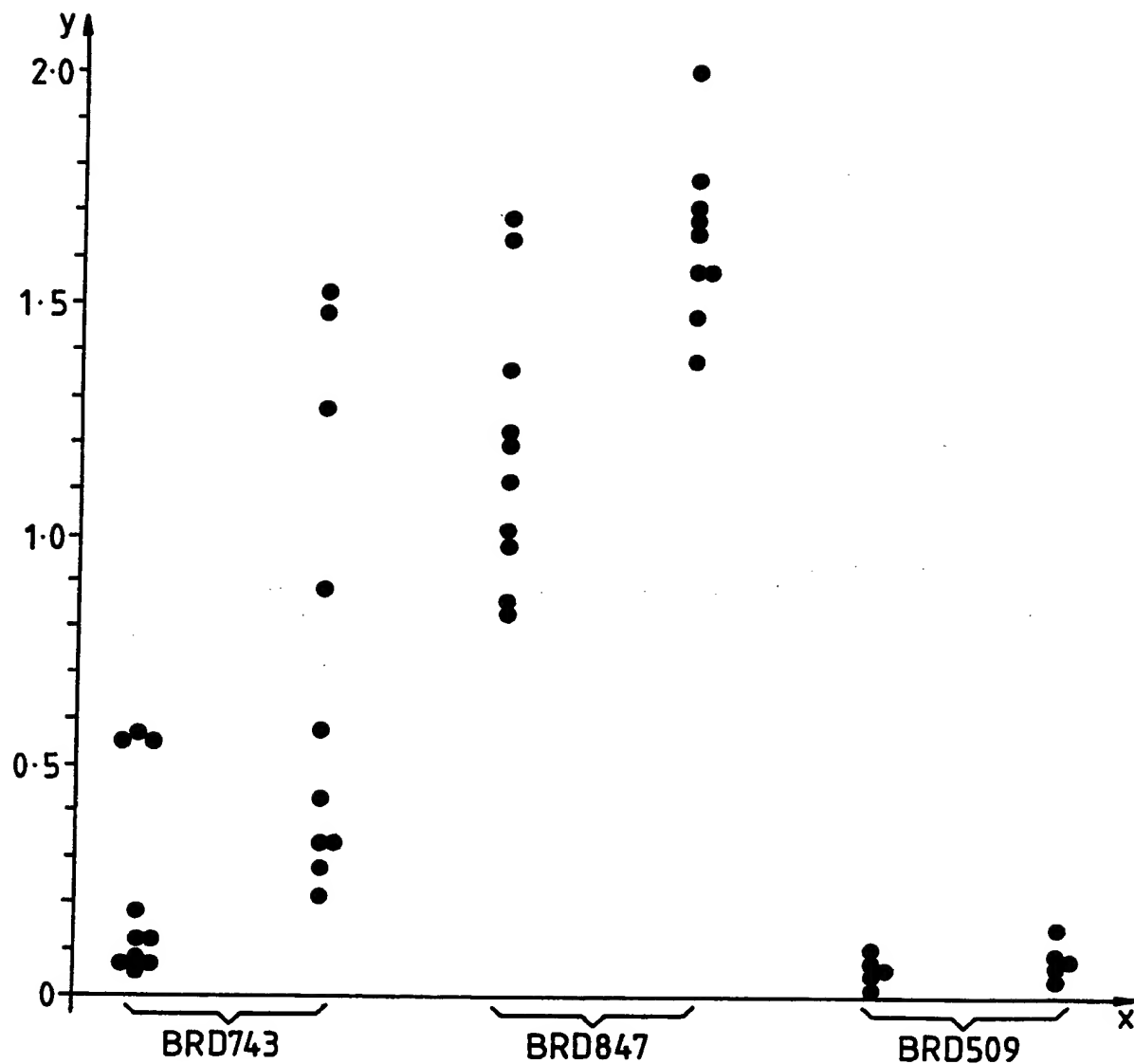


Fig.4.

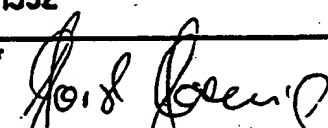


*Fig.5.*

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 92/00387

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/74; A61K39/10;	C12N15/70; C12N1/21;	C12N15/31; A61K39/112 /(C12N1/21, C12R1:42)
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C12N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y	EP,A,0 285 152 (BOEHRINGER MANNHEIM) 5 October 1988	1-12
Y	RES. MICROBIOL. vol. 141, no. 7-8, 1990, INSTITUT PASTEUR, ELSEVIER, PARIS; pages 769 - 773; N.F. FAIRWEATHER ET AL.: 'Use of live attenuated bacteria to stimulate immunity' International symposium on oral immunization using recombinant bacteria, Munich, Germany, June 6-7, 1990	1-12
<p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
04 JUNE 1992	09 JUN 1992	
International Searching Authority	Signature of Authorized Officer	
EUR PEAN PATENT FFICE	HORNIG H. 	

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	<p>INFECTION AND IMMUNITY vol. 58, no. 5, May 1990, AM. SOC. MICROBIOL., BALTIMORE, US; pages 1323 - 1326; N.F. FAIRWEATHER ET AL.: 'Oral vaccination of mice against tetanus by use of a live attenuated salmonella carrier'</p> <p>---</p>	1-12
Y	<p>MOLEC. MICROBIOL. vol. 4, no. 10, October 1990, BLACKWELL SCI. PUB., OXFORD, UK; pages 1753 - 1763; A.I. BELL ET AL.: 'Molecular genetic analysis of an FNR-dependent anaerobically inducible escherichia coli promoter' cited in the application</p> <p>---</p>	1-12
Y	<p>MOLEC. MICROBIOL. vol. 2, no. 4, April 1988, BLACKWELL SCI. PUB., OXFORD, UK; pages 527 - 530; P.S. JAYARAMAN ET AL.: 'The nirB promoter of escherichia coli: location of nucleotide sequences essential for regulation by oxygen, the FNR protein and nitrite'</p> <p>---</p>	1-12
Y	<p>EP,A,0 357 208 (SMITHKLINE BECKMANN CORPORATION) 7 March 1990</p> <p>---</p>	1-9,11, 12
Y	<p>EP,A,0 400 958 (THE WELLCOME FOUNDATION LIMITED) 5 December 1990 cited in the application</p> <p>---</p>	1-9,11, 12
Y	<p>EP,A,0 322 237 (THE WELLCOME FOUNDATION LIMITED) 28 June 1989 cited in the application</p> <p>---</p>	1-9,11, 12

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO. GB 9200387  
SA 57302**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 04/06/92

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		JP-T- 2502785	06-09-90